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Award Number: W81XWH-05-1-0342

TITLE: EPR Assembly of Microgel for FRET Imaging of Breast Cancer

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REPORT DATE: April 2008

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01/04/08	Final	1 APR 2005 - 31 MAR 2008

4. TITLE AND SUBTITLE
EPR Assembly of Microgel for FRET Imaging of Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0342

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Rutgers the State University of New Jersey
New Brunswick, New Jersey 08901-8559

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of this project is to develop a new process for identifying breast cancer. This process should be at least as convenient as mammography for the patient, more sensitive for detection of the smallest and earliest tumors, and more accurate in distinguishing tumors from normal tissues. First the tumor site is marked by entrapment of a polymer-PNA (peptide nucleic acid) conjugate due to EPR effect. The complementary PNA sequence labeled with fluorescein (PNA-5'-Fluorescein) is administered next. By itself, this PNA-fluorescein conjugate would have minimal retention in the tumors because of its smaller size. However, the PNA-Fluorescein conjugates are retained in the tumor tissues due to the Watson-Crick base pairing between the complementary PNA sequences, which non-covalently link the fluorescein group as a polymer-PNA:complementaryPNA-Fluorescein complex. The fluorescence signal is detected non-invasively. In the three years, we have established animal model, developed a non-invasive procedure to detect and measure enhanced permeability and retention effect, and investigated EPR effect exerted by polymers of different molecular size. We have completed the synthesis of polymer-PNA conjugates and currently investigating their network formation in tumors.

15. SUBJECT TERMS
Fluorescence, Enhanced Permeability and Retention (EPR), Poly(ethylene glycol), 4T1 Cells.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	14	USAMRMC

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1. INTRODUCTION

The purpose of this project is to develop a method to detect breast cancer with greatest possible accuracy at smallest possible size. The method should give least stress and inconvenience to the patient. We have proposed to combine different techniques into a three-step process to accomplish this goal (Figure 1).

1. The first goal is to place a "marker" on tumor. For this purpose, we will use the "enhanced permeability and retention (EPR)" effect[1-3], which might be universal for all tumors. The markers developed herein will be optimized for their ability to accumulate in tumor but not in normal tissues. We propose to use polymer-5'-PNA (peptide nucleic acid)[4-6] conjugates that will accumulates in tumors due to the EPR effect.
2. The second goal is target the signaling moiety selectively to the tumors. This is to be accomplished by using dye-labeled complementary PNA sequence. This complementary sequence will have minimal retention in the body or tumors on account of its smaller size. However, in this case, the dye-labeled PNA conjugates will be retained in tumors because of the Watson-Crick base pairing between the complementary PNA sequences. The specific base pairing between the complementary sequences will link the signaling (dye) moiety to polymer-PNA:complementary-PNA-dye complex.
3. The third goal is to detect the signal non-invasively.

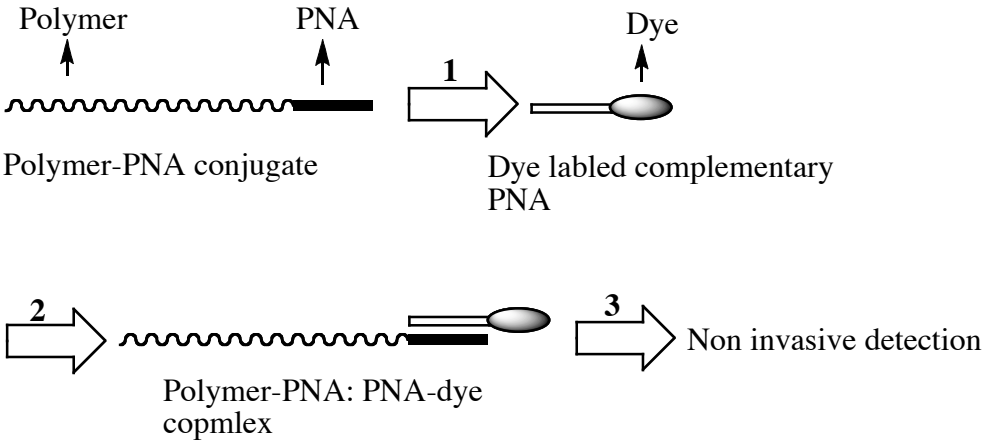


Figure 1. A schematic presentation of three-step process for non-invasive detection of breast cancer. Polymer-PNA conjugate is administered in the first step, which accumulates passively in the tumor tissues. Dye labeled complementary PNA is administered next, which is cleared from the body but not tumors due to the formation of polymer-PNA:PNA-dye complex. Signals are detected non-invasively.

In the first year of grant, an animal model for breast cancer was established whereas in the second year we developed a non-invasive procedure to detect and measure tumor accumulation of polymeric fluorescent agents (See the

reports submitted in May 2006 & May 2007). In the third year we have investigated the EPR effect exerted by polymeric fluorescent agents of different molecular size so as to optimize their tumor accumulation properties.

2. BODY

Prodrugs / imaging agents can be targeted to tumors by passive processes. This is achieved by covalent attachment of drugs or imaging moieties to larger molecules (synthetic or biopolymers) or nanoparticles (liposomes, nanospheres) that act as inert carriers[7-8]. The underlying concept behind this is to take advantage of the enhanced permeability and retention (EPR) effect elucidated by Maeda and coworkers[1-3] (See below).

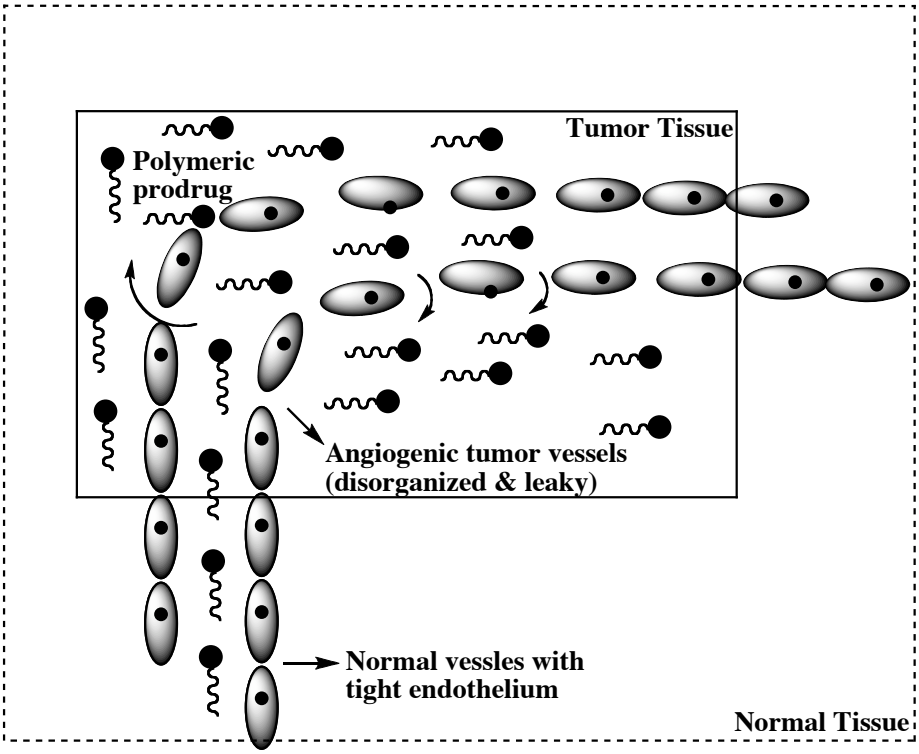


Figure 2. Schematic presentation of enhanced permeability and retention (EPR) effect. The polymers, nanoparticles, and liposomes penetrate into the tumor tissues, which has leaky tumor vessels unlike the normal tissues. These nanoparticulate systems beyond certain molecular size are retained in tumors because of its impaired lymphatic drainage. EPR effect has been used to passively target drugs/imaging agents selectively to tumors.

Angiogenesis is induced in tumors to accommodate their ever-increasing demand for nutrition and oxygen as the tumor cells multiply and cluster together to reach the size of 2-3 nm[9] (Figure 2). The neovasculature in tumors differs greatly from the microscopic anatomical architecture of normal tissue. For instance, the blood vessels in tumors are irregular in shape, dilated, leaky or defective, and the endothelial cells are poorly aligned or disorganized with large fenestrations. The perivascular cells and the smooth muscle layers are frequently

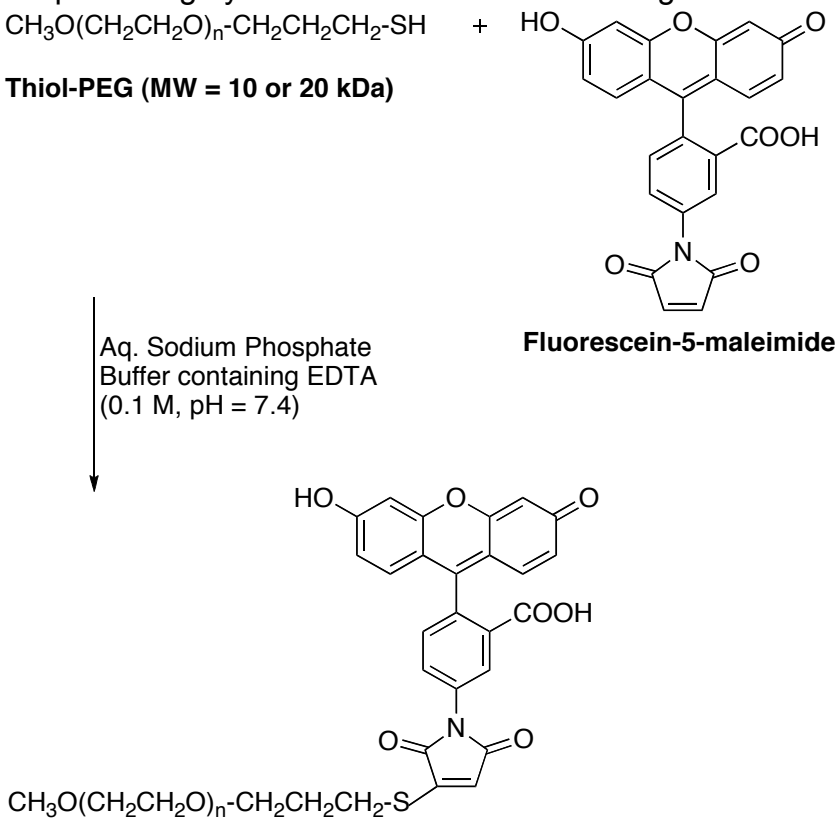
absent or abnormal in the vascular wall. Tumor vessels have wide lumens, whereas tumor tissues have poor lymphatic drainage. This results into extensive leakage of blood plasma components such as macromolecules, nanoparticles, and lipidic particles in tumor tissues. These macromolecules and nanoparticles are retained in tumors due to the slow venous return in tumor tissues and poor lymphatic drainage. This phenomenon is known as the EPR effect. The pore size of tumor microvessels may vary from 100 to 1200 nm in diameter whereas the tight junctions between endothelial cells of microvessels are mostly under 2 nm (exceptions: ~6 nm in kidney, liver and spleen). Macromolecular carriers, on the other hand, have hydrodynamic radii in the range of 2-10 nm, which allows their extravasation into tumor tissues and not in normal tissue. It must be mentioned that enhanced permeability is true for smaller molecules also. However, large molecules with molecular weight of ≥ 40 kDa show decreased clearance from tumors (i.e., they also demonstrate enhanced retention). As a result, small molecules are rapidly cleared from the tumor interstitium, whereas the large molecules are retained. Thus, it is a combination of enhanced permeability and impaired clearance that account for the accumulation of macromolecular prodrugs in tumors due to the EPR effect. It should be cautioned that molecular weight is absolutely important, but not the sole criterion for predicting the molecule's biodistribution. The chemical nature of polymer, as well as shape and conformation in water, may also influence its molecular size.

The EPR effect can be observed for proteins with molecular weight greater than 50 kDa. The molecular size of proteins cannot however be related to polyethylene glycol polymers because proteins possess a very compact and globular structure whereas polymers display a coiled/extended conformation[10]. Poly(ethylene glycol) polymers has higher molecular volume as compared to proteins with same molecular weight due to random conformation and ability to coordinate water molecules (three per monomeric unit). This water cloud gives high hydrodynamic volume to PEG that makes it difficult to compare sizes among proteins, polymers or polymer conjugates.

The optimization of EPR properties of polymers is key to the successful implementation of the present project. In the first two years of the grant we have established an animal model to investigate breast cancer and a non-invasive procedure to detect and measure accumulation of polymeric fluorescent agents in tumors through EPR. In the current year, we have investigated the passive targeting accomplished by polymers of different molecular size through EPR. A brief summary of the work accomplished in the third year of the grant is given below.

Task 1: Synthesis and characterization of polymeric fluorescent agents of different molecular sizes*:* Polymeric fluorescent agents were obtained by reacting poly(ethylene glycol) polymers (PEG) of different molecular weights with fluorescein (reporter group) as shown in Scheme 1. Briefly, thiol-functionalized PEG's (10 & 20 kDa) were reacted with 5-equivalents of fluorescein-5-maleimide

in sodium phosphate buffer containing EDTA (0.1 M, pH = 7.4). Similarly, amine-functionalized PEG (40 kDa) was reacted with fluorescein succinimide ester in sodium phosphate buffer (0.1 M, pH = 8.0). The reaction mixtures were stirred at room temperature for overnight period and products were obtained after purification by gel-permeation chromatography (GPC) on Sephadex G-50 column. The percentage yields obtained were in the range of 72-79%.



Scheme 1. General synthetic scheme to prepare fluorescein-labeled PEG polymers (10 and 20 kDa). The PEG-fluorescein (40 kDa) was obtained by analogous procedure. PEG-NH2 was reacted with fluorescein succinimidyl ester at pH 8.0.

The product purities were estimated on Waters Breeze GPC system equipped with refractive index and UV detectors (λ_{abs} = 480 nm). Analysis was performed on Waters 1000 column (7.8 x 300 mm) and double-distilled water was used as mobile phase (flow rate: 1.0 mL/min). The sample concentration was 2 mg/mL whereas the injection volume was 0.2 mL. There was significant difference between the retention time observed for labeled polymers and the unlabeled polymer. The retention times for 10, 20, and 40 kDa products were obtained as 8.2, 7.4, and 5.7 minutes respectively whereas the retention times for corresponding starting materials were obtained as 8.9, 8.3, and 7.6 minutes respectively (Figure 3a-f). All products were obtained in high purity (>99%) and no free fluorescein was detected during the GPC analysis.

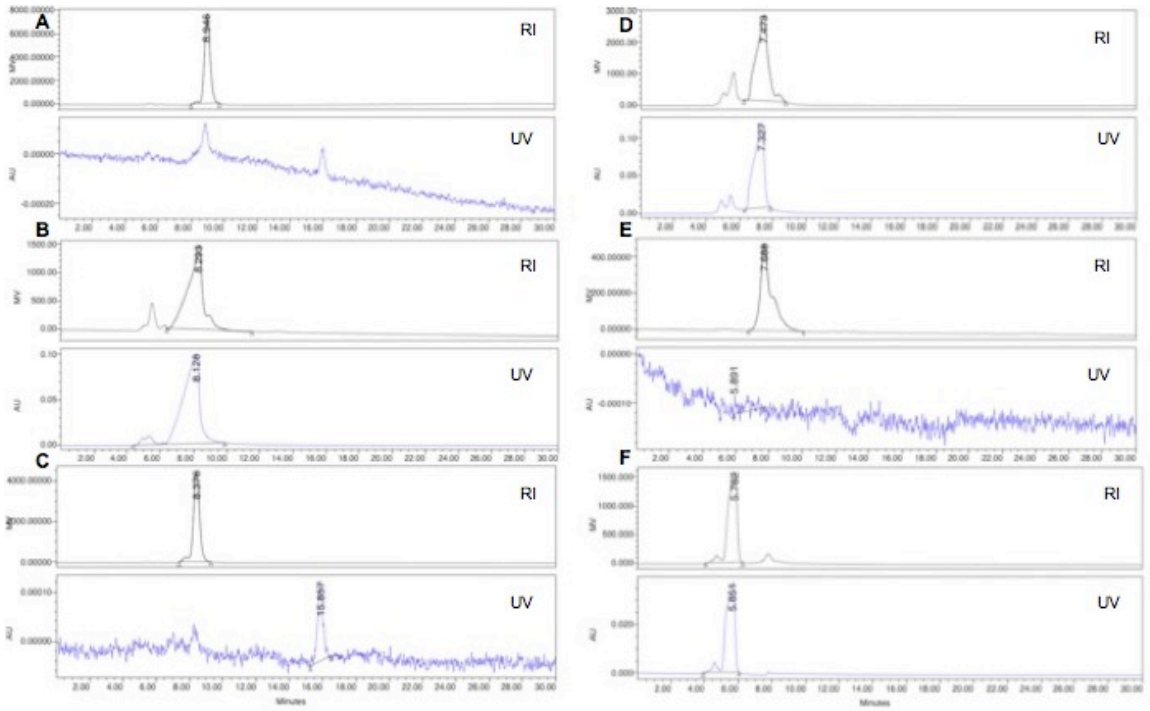


Figure 3. GPC profile for PEG polymers: (A) PEG (10 kDa); (B0 PEG-Fluorescein (10 kDa); (C) PEG (20 kDa); (D) PEG-Fluorescein (20 kDa); (E) PEG (40 kDa); (F) PEG-Fluorescein (40 kDa). RI = Refractive Index detector; UV = UV absorption detector (λ_{abs} = 480 nm).

The analysis was performed on Voyager-DE Pro mass spectrometer in Linear and positive mode using sinapinic acid as matrix. The sample concentration were 1mg /100 μL and water:acetonitrile:trifluoroacetic acid (50:50+0.3) was used a solvent system. The sample solution was mixed with matrix solution and spotted (2 μL) on 100 well MALDI-TOF plates. The spots were dried before the analyses. The molecular weights obtained for 10, 20, and 40 kDa starting material were 10377, 20822, and 42668 Da respectively whereas the molecular weights for corresponding products were obtained as 10973, 22332, and 43554 Da respectively. The MALDI-TOF spectra of PEG-Flurescein are shown in Figure 4 (See below).

Conclusion: Polymeric fluorescent agents of different molecular sizes (10, 20, and 40 kDa) were obtained in high yield and purity for *in vivo* investigations. They were characterized by satisfactory mass spectrometric data.

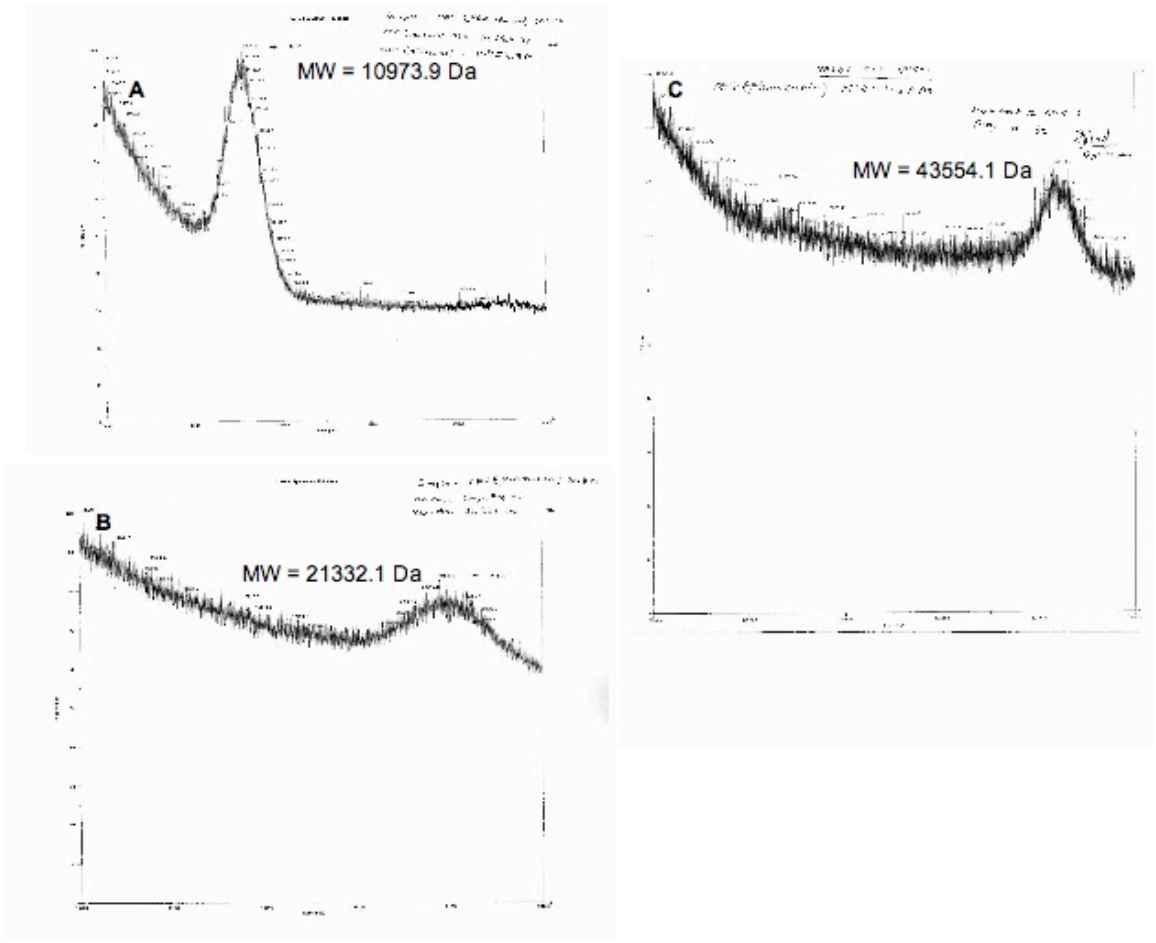


Figure 4. MALDI-TOF spectra of fluorescein labeled PEG polymers: (A) 10 kDa; (B) 20 kDa; (C) 40 kDa. Sinapinic acid was used as matrix and solvent system used was acetonitrile:water:TFA:::50:50:0.3 (v/v/v).

Task 2: Investigation of EPR effect exerted by polymeric fluorescent agents of different molecular sizes (by non-invasive skinskan): 8-weeks old Female Balb/c mice were obtained from Jackson Laboratories and six animals were used for each polymer. They were shaved and inoculated subcutaneously with 4T1 cells (10^7/mouse) into the dorsal area. Seven days after cell injection, animals have their hair removed again. One day after hair removal, 0.1 ml of polymer-fluorescein was injected through tail lateral vein. Equimolar polymer solutions were prepared in saline by dissolving 5mg/mL (10 kDa), 10 mg/mL (20 kDa), and 20 mg/mL (40 kDa) respectively. A fiber optic-based skinskan fluorimeter (Jobin Yuvon / Spex Industries) was used to collect *in vivo* fluorescence spectra from the tumor and the control site present on the skin of live mice (Figure 5a-d). The fluorescence spectra were collected at different time points (0.5, 1, 2, 3, 4, 5, 6, and 24 hours). Fluorescence spectra were recorded on the skin of untreated mice and no significant emission was notice in the region of interest (500-600 nm).

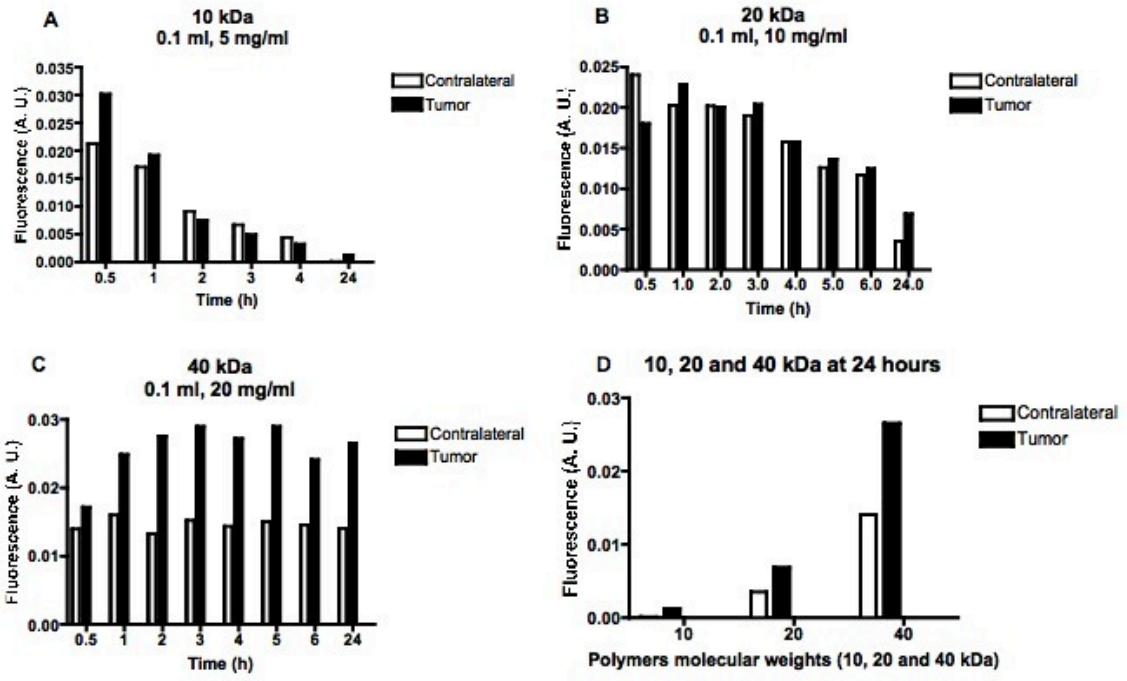


Figure 5. EPR effect shown by fluorescein labeled PEG polymers of different molecular sizes: (A) 10 kDa; (B) 20 kDa; (C) 40 kDa; (D) Comparative tumor accumulation of three polymers at 24 hours after injection.

Results show that 10 kDa polymer concentrations peaked in 0.5 hours before decreasing gradually (Figure 5a). Fluorescence measured from tumor site was slightly higher than the control site. The fluorescence from control site falls to background level (complete clearance) after 24 hours but tumor site showed measurable fluorescence because of polymer accumulation in tumors. The 20-kDa polymer concentrations peaked in 1-3 hours before decreasing gradually (Figure 5b). The fluorescence measured from tumor site was moderately higher than fluorescence measured from the control site. The difference between the two became significant after 24 hours as the polymer was cleared from the body and not tumors. The 40-kDa polymer concentrations peaked in 3-5 hours (Figure 5c). The fluorescence measured from tumor site was significantly higher than the control site and they the difference between the two increased after 24 hours. The fluorescence measured from tumor site after 24 hours were 6% higher than the control site for 10-kDa, 50% higher for 20-kDa, and 53-54% higher for 40-kDa (Figure 5d). Thus, the three polymers showed tumor retention in the order 40 > 20 > 10-kDa.

After 24 hours animals were sacrificed to collect various tissue samples. The tissue samples were homogenized to extract the polymeric fluorescent agents and fluorescence was measured on Fluorescence plate reader. The amount of polymeric fluorescent agent present in tissues was quantified by using a standard curve (Figure 6). The highest concentration of polymers was found in tumors followed by liver, kidney, muscle, and spleen. The tumor retention of three polymers were found again in the order 40 > 20 > 10-kDa. Thus, the data

obtained from plate reader validated the results obtained from non-invasive skinskan.

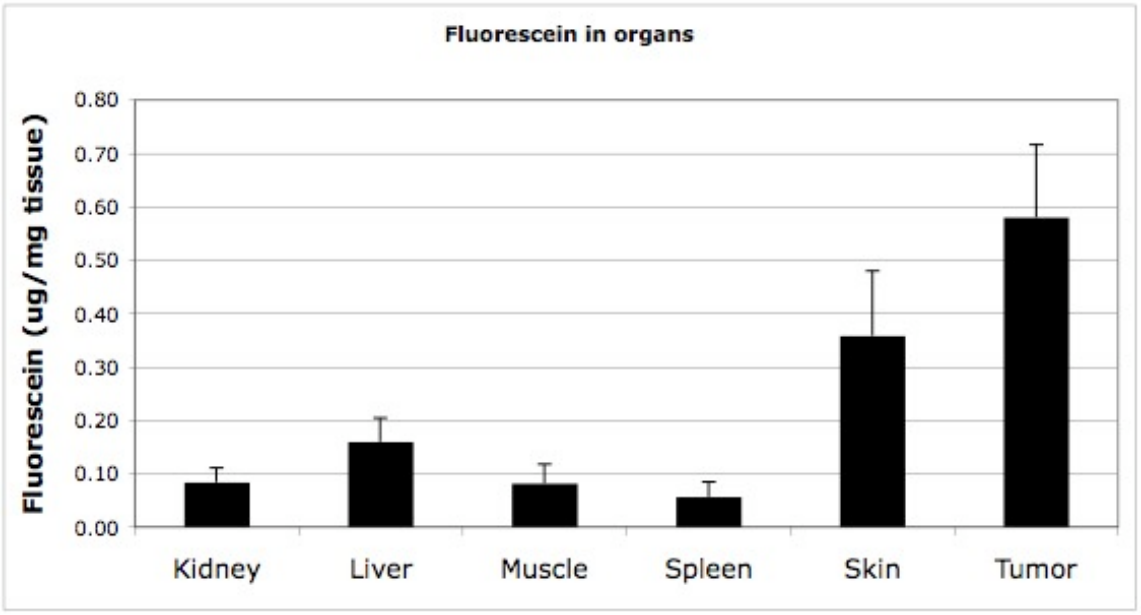


Figure 6. Biodistribution of labeled PEG polymer (20 kDa) in different tissues. The 40 kDa labeled PEG polymer also showed similar biodistribution pattern (not shown).

Conclusion: Passive targeting properties of polymeric fluorescent agents with different molecular size (10, 20, and 40 kDa) were investigated. It was observed that the passive accumulation of polymers in the tumor tissues increased with increasing molecular weights. The molecular weight of 20 kDa was found optimum to obtain significant EPR effect. The conventional invasive procedure (plate reader) was used to validate the results obtained from non-invasive skinskan.

Task 3: Investigation of EPR effect exerted by polymeric fluorescent agents of different molecular sizes (IVIS 100 imaging system): 8-weeks old Female Balb/c mice were used for this study (six animals for each polymer). They were treated and inoculated with 4T1 cells as describe above for skinskan studies. 0.1 ml of polymer-fluorescein was injected through tail lateral vein. Equimolar polymer solutions were prepared in saline by dissolving 5mg/mL (10 kDa), 10 mg/mL (20 kDa), and 20 mg/mL (40 kDa) respectively. Animals were immediately anesthetized with isofurane and whole body image was investigated using IVIS 100 imaging system. The imaging experiments for each polymer group were carried out in two sets (three mice treated with polymer and two untreated mice for control studies). The whole body images were recorded at 0.5, 6, 24, 48, and 75 hours.

Accumulation of fluorescein-labeled PEG (40 kDa) was clearly evident at 6 hours (Figure 7). Polymers were found retained in tumors for up to 72 hours. Polymeric fluorescent agent with molecular size of 10 kDa and 20 kDa also showed retention in tumors at 24 hours (not shown). The high background fluorescence

was evident around the neck region. The problem of high background fluorescence and artifacts can be excluded by using near infrared fluorescence probes. The background fluorescence (autofluorescence) from biological samples is negligible in near IR region and maximum light penetration is possible in this region of spectrum.

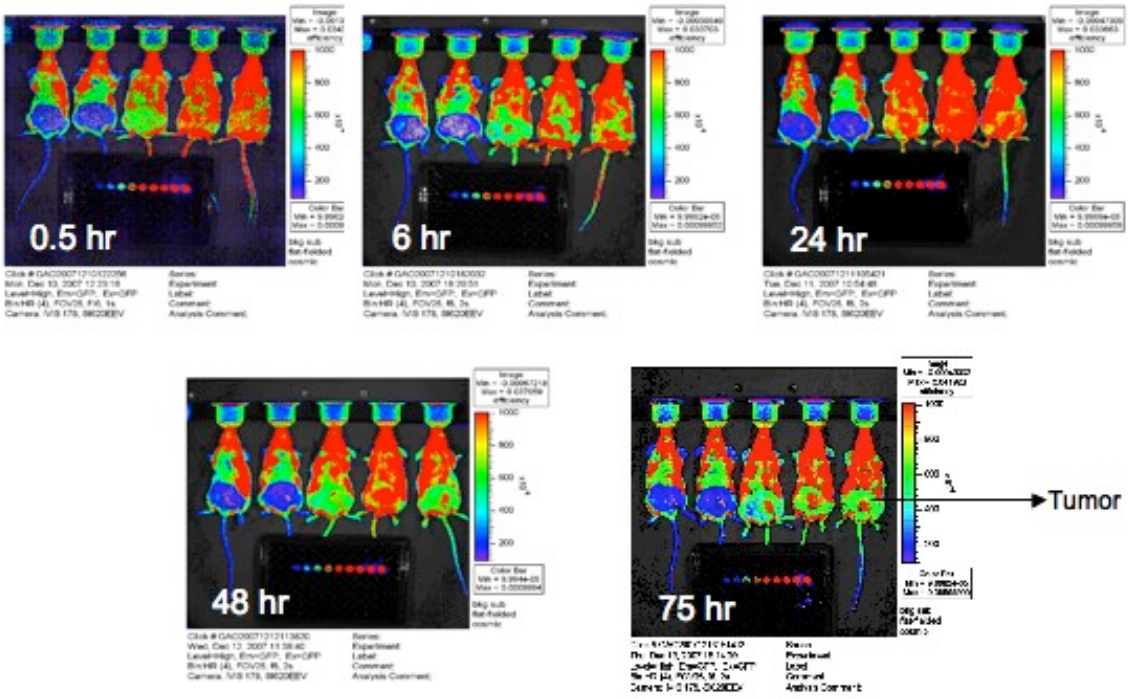


Figure 7. Whole body images of mice treated with labeled PEG polymer (40 kDa). The first two mice from left are untreated for control studies whereas the remaining three mice were given 0.1 ml of polymer (20 mg/mL) through intravenous injection. The images obtained for mice injected with 10 and 20 kDa labeled polymers are not shown here.

Conclusion: The whole body images were recorded by IVIS camera to investigate passive targeting properties of polymeric fluorescent agents with different molecular size (10, 20, and 40 kDa). The results obtained from IVIS camera were in agreement with the earlier results obtained from non-invasive skinskan. The imaging experiments need further standardization with near infrared probes. Use of fluorescence groups emitting in visible region may not be useful due to the high background fluorescence from biological samples.

3. KEY RESEARCH ACCOMPLISHMENTS

(a) Polymeric fluorescent agents of different molecular size were prepared and characterized in high yield and purity for *in vivo* studies.
(b) A non-invasive skinskan procedure was established to detect and measure passive accumulation (enhanced permeability and retention effect) of polymeric fluorescent agents in the tumor tissues.
(c) Tumor accumulation of polymeric fluorescent agents with different molecular size (10, 20, and 40 kDa) was investigated by non-invasive

skinskan and IVIS imaging system to investigate the influence of molecular weight on their passive targeting properties.

(d) Results obtained show that molecular size of PEG does influence their passive targeting properties. The EPR effect shown by the polymers of different molecular weights were in the order 40 > 20 > 10 kDa.

(e) PEG because of their high hydrodynamic radius exerts EPR at considerably low molecular weights. In present studies, PEG with 20 kDa showed significant EPR effect. This is contrary to proteins, which show EPR effect at molecular weight <50 kDa.

(f) The results obtained by non-invasive skinskan and IVIS imaging system were validated by conventional procedure (invasive studies with plate reader).

(g) Our efforts are now directed towards the formation of polymer-PNA:complementary-PNA-dye complex into the tumor tissues so as to passively target imaging agents selectively to tumors.

4. REPORTABLE OUTCOMES

(a) A non-invasive procedure has been developed to detect and measure passive accumulation of polymeric fluorescent agent into the tumor tissues.

(b) The influence of molecular size on passive accumulation of polymeric fluorescent agent due to EPR effect has been investigated.

(c) **Posters:** Following papers have been presented or accepted for presentation as posters in conferences;

  (i) Yashveer Singh, Dayuan Gao, Peidi Hu, **Stanley Stein**, Patrick J. Sinko, Investigation of enhanced permeability and retention in tumors by using non-invasive techniques, *PharmSci Supplement W4280, 2007 American Association of Pharmaceutical Scientists Annual Meeting and Exposition*, San Diego, CA, USA, 11-15 November, 2007.

  (ii) Yashveer Singh, **Stanley Stein**, Dayuan Gao, and Patrick J. Sinko, Polymer assembly in tumors to detect breast cancer, Vth Era of Hope Conference, Baltimore, MD, June 25-28, 2008 (Accepted for presentation).

  (iii) Yashveer Singh, **Stanley Stein**, Dayuan Gao, and Patrick J. Sinko, Non-invasive detection of enhanced permeability and retention of polymers in tumors, Vth Era of Hope Conference, Baltimore, MD, June 25-28, 2008 (Accepted for presentation).

  (iv) Yashveer Singh, **Stanley Stein,** Dayuan Gao, and Patrick J. Sinko, Using enhanced permeability and retention effect for targeting of fluorescent agent and signal amplification, Vth Era of Hope Conference, Baltimore, MD, June 25-28, 2008 (Accepted for presentation).

(d) **Talk:** Patrick J. Sinko, Yashveer Singh, Dayuan Gao, and **Stanley Stein**, Non-invasive procedure to detect polymeric fluorescent agents targeted to

Tumors, Materials Research Society – Internationational Materials Research Conference, June 9-12, 2008, Chongqing, China.

(e) **Manuscript:** Yashveer Singh, Dayuan Gao, **Stanley Stein**, Patrick J. Sinko, Non-invasive detection of enhanced permeability and retention of polymers in tumors, *J. Pharmacol. Exp. Ther.* **2008** (To be submitted shortly).

5. CONCLUSIONS

In the three years of grants we have successfully established an animal model to investigate breast cancer and a non-invasive procedure to detect and measure polymeric anticancer drugs and fluorescent agents targeted selectively to tumors. We have also investigated the influence of molecular size of poly(ethylene glycol) polymers on their passive targeting properties. It has been shown that unlike proteins, which show enhanced permeability and retention (EPR) effect at molecular weight <50 kDa, the PEG polymers show significant EPR effect even at molecular weight <20 kDa. The development of non-invasive procedure to detect and measure EPR effect is a noteworthy accomplishment because it may lead to the development of non-invasive, rapid, convenient, and cost effective procedure for screening of polymeric anticancer drugs/fluorescent agents targeted passively to tumors.

We have completed the synthesis and characterization of PEG-PNA conjugates and dye-labeled complementary PNA sequence and investigating their hybridization behavior in tumors.

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